

California State University, San Bernardino

CSUSB ScholarWorks

Theses Digitization Project

John M. Pfau Library

2005

Thyroid hormone-regulated skeletal muscle Glut4 glucose transporter trafficking during fasting in diet-induced obesity and insulin resistance

Lucy Soo Yon Jun

Follow this and additional works at: <https://scholarworks.lib.csusb.edu/etd-project>



Part of the [Endocrinology, Diabetes, and Metabolism Commons](#)

Recommended Citation

Jun, Lucy Soo Yon, "Thyroid hormone-regulated skeletal muscle Glut4 glucose transporter trafficking during fasting in diet-induced obesity and insulin resistance" (2005). *Theses Digitization Project*. 2869. <https://scholarworks.lib.csusb.edu/etd-project/2869>

This Thesis is brought to you for free and open access by the John M. Pfau Library at CSUSB ScholarWorks. It has been accepted for inclusion in Theses Digitization Project by an authorized administrator of CSUSB ScholarWorks. For more information, please contact scholarworks@csusb.edu.

THYROID HORMONE-REGULATED SKELETAL MUSCLE GLUT4 GLUCOSE
TRANSPORTER TRAFFICKING DURING FASTING IN DIET-INDUCED
OBESITY AND INSULIN RESISTANCE

A Thesis
Presented to the
Faculty of
California State University
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Sciences
in
Biology

by
Lucy Soo Yon Jun
June 2005


THYROID HORMONE-REGULATED SKELETAL MUSCLE GLUT4 GLUCOSE
TRANSPORTER TRAFFICKING DURING FASTING IN DIET-INDUCED
OBESITY AND INSULIN RESISTANCE

A Thesis
Presented to the
Faculty of
California State University
San Bernardino


by
Lucy Soo Yon Jun


June 2005

Approved by:


Richard Fehn, /Chair, Biology

6/6/05
Date


James Ferrari


Anthony Metcalf

ABSTRACT

The independent versus joint effects of insulin and thyroid hormone (T_3) on soleus muscle glucose transport protein (GLUT4) trafficking were assessed in diet-induced obese (DIO) insulin resistant rats, a model for type 2 diabetes mellitus. Wistar and Sprague-Dawley (SD) rats were compared after high-fat (HF) feeding during which Wistar rats gained weight faster and achieved higher final body weights than SD rats. Oral glucose tolerance tests revealed insulin resistance in the Wistar rats and these animals were subsequently given streptozotocin to block insulin production followed by insulin injections at doses of 1 IU/kg BW to induce normoglycemia or a half dose of 0.5 IU/kg to mimic fasting serum insulin levels. Animals were fed or fasted overnight to manipulate serum T_3 concentrations. Fasting lowered serum T_3 in animals receiving full insulin replacement but not in low insulin dose animals. GLUT4 concentrations were unchanged by treatments which confirmed insulin resistance. However, serum glucose concentrations were still reduced by the high insulin dose indicating a persistent peripheral insulin response. This study revealed a role for insulin in the regulation of serum T_3 that offers a novel pathway for

integrating the daily insulin concentrations for the long-term regulation of skeletal muscle GLUT4 expression by T_3 .

ACKNOWLEDGEMENTS

I would like to thank Dr. Richard Fehn for his guidance, patience and unending support throughout my thesis project. From the very first experiments, to the data analyses and finally the writing of my thesis, Dr. Fehn continually renewed my faith in science and in my abilities as an investigator while sharing in the sense of wonderment at the intricacies of integrative physiology. This work would not have been possible from its inception without Dr. Fehn and his profound dedication as a mentor and as a consummate physiologist. I am therefore most indebted to him for this work.

My thesis advisory committee members Drs. James Ferrari and Anthony Metcalf gave valuable time and thoughtful feedback on the project and my written drafts. I would like to thank them both for their insightful guidance which helped shape my writing throughout my thesis.

I also like to thank Matthew Ricci, Ph.D. and Vice President of Operations at Research Diets, Inc. for generously donating the rodent high fat chow that was used to feed the Wistar animals throughout this study. Dr.

Ricci's contribution came at a crucial time in my project and was instrumental in its timely completion.

Finally, I would like to thank the Biology Department at California State University San Bernardino, the Associated Students, Inc. and the California Pre-Doctoral Program for their support and funding throughout my thesis project.

TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGEMENTS	v
CHAPTER ONE: INTRODUCTION	
Obesity and Type 2 Diabetes in the 21 st Century	1
Lipids and Insulin Resistance	3
Glucose Homeostasis	4
Fasting and Nonfasting <i>In Vivo</i> Metabolism	6
Nutrient Regulation by Leptin and Thyroid Hormone	8
Purpose of the Thesis	15
Pilot Study on Hormone Interactions	15
Independent versus Joint Effects of Insulin and T ₃	16
CHAPTER TWO: MATERIALS AND METHODS	
Animals	18
Experimental Treatments	19
Tissue Collection	21
<i>In Vivo</i> Metabolic Analyses	22
Serum Hormone and Glucose Assays	22
Soleus Muscle GLUT4 Quantification	23
Data Analyses	27

CHAPTER THREE: RESULTS

High-Fat Diet and Weight Gain	28
<i>In Vivo</i> Metabolic Analyses	28
Oral Glucose Tolerance Tests	29
Serum T ₃ , Insulin and Glucose Levels	30
Plasma Membrane GLUT4 Concentrations	32
CHAPTER FOUR: DISCUSSION	33
APPENDIX A: FIGURES	46
APPENDIX B: PILOT STUDY DATA	58
APPENDIX C: TABLES	61
LIST OF REFERENCES	66

CHAPTER ONE

INTRODUCTION

Obesity and Type 2 Diabetes in the 21st Century

As this country enters the new millennium, it faces a precipitous rise in the rate obesity and its co-morbid conditions. Over the past two decades, the prevalence of obesity in the United States has risen by about 50% and shows no signs of abating (Rippe et al, 2001). The prevalence and severity of obesity affects both men and women and virtually all ethnic groups. Childhood and adolescent obesity also continues to rise rapidly (Troiano, et al, 1995; NIH, 1996). In a recent study by the University of Southern California, it was estimated that the annual cost of 16-17 year-olds in hospitals due to obesity has reached \$127 million per year (Goran & Cruz, 2003). An estimated 300,000 deaths annually are attributable to obesity in the United States, thus ranking obesity-related factors second only to smoking among the leading causes of preventable death in adult Americans (Rippe et al, 2001).

While it has been hotly debated whether this rising problem with obesity is due to genetic or environmental

factors, the scientific community has reached a consensus that while genes make certain individuals more susceptible than others, behavior has played a crucial role in the population's attitude toward nutrition, exercise and fitness, exacerbating this epidemic. The occurrence of obesity is, therefore, influenced by a complex interaction of genetic, environmental and behavioral factors (Rippe et al, 2001; Pi-Xunyer, 2002). The major difference in lifestyle between now and forty years ago is the disproportionately high intake of calorie-rich foods in the absence of the required energy expenditure to burn the excess fuel. These factors are compounded in individuals who display the propensity for weight gain even though they eat as much as another, less susceptible individual (McGarry, 2001). The earlier stages of this type of metabolic dysregulation is called Metabolic Syndrome X, which occurs when an individual displays hyperinsulinemia with concomitant hyperglycemia due to insulin resistance of peripheral tissues like the liver and the skeletal muscle. Type 2 diabetes mellitus occurs when pancreatic beta cell failure takes place and the individual no longer synthesizes or secretes endogenous insulin (McGarry, 2001). Type 2 diabetes has long been labeled as a disease of

inadequate glycemic control that is accompanied by obesity. However, it has recently come to light that defects in fatty acid metabolism may be the actual cause of the insulin resistance characteristic of type 2 diabetes, strengthening the connection between obesity and insulin resistance (McGarry, 2001; Kuhlmann et al, 2003).

Lipids and Insulin Resistance

Dennis McGarry introduced a novel idea that lipid accumulation inside the skeletal muscle, or intramyocellular lipids (IMCL), plays a major role in the onset of insulin resistance and hyperglycemia (McGarry, 2003). While the exact mechanisms have yet to be elucidated, this hypothesis suggests a role for fatty acids in the derangement of the regulatory pathways that control everything from insulin transduction to transcription factors in the skeletal muscle, adipocytes, vascular endothelium and the liver (D'Alessandro et al, 2000). Because it is a critical site of glucose disposal, and because it constitutes the greatest tissue mass, skeletal muscle is considered the main contributor to hyperglycemia due to defective insulin-controlled glucose regulation (Peterson & Shulman, 2002). Individuals who are

predisposed to obesity have been found to have a decreased capacity to oxidize fatty acids in their skeletal muscle which leads to the accumulation of IMCL and the subsequent inability of the muscle to respond to insulin signaling (Kim et al, 1999; Tremblay et al, 2001). Excess IMCL accumulation prevents the muscle from taking up and utilizing glucose through the normal pathways, thus leading to its accumulation in the blood and subsequent hyperglycemia (Laybutt et al, 1999). Insulin resistance in the muscle, therefore, is the root of defective glucose homeostasis. Skeletal muscle insulin resistance becomes further exacerbated by hepatic insulin resistance, which causes excessive glucose release into the bloodstream (Peterson & Shulman, 2002; Standaert et al, 2002).

Glucose Homeostasis

Since the recent focus of diabetes research has been on fat metabolism and how it affects a tissue's ability to regulate glucose, it is important to study the mechanism by which glucose regulation takes place. One such mechanism addresses the means by which the hydrophilic glucose molecule is able to cross cellular membranes. The chemical nature of this molecule requires that it be transported

though the hydrophobic domain of membranes in order to pass first into the cytoplasm by crossing the plasma membrane, and then to different subcellular compartments by crossing organellar membranes.

There are seven identified glucose transporter proteins, GLUT1 through GLUT7. Different tissues have characteristic expression patterns of glucose transporter proteins that serve as controlled gates to take glucose in and out of the cell depending on serum glucose concentrations. The skeletal muscle has GLUT4 proteins that respond to insulin by increasing their numbers in the plasma membrane (Figure 1). High serum glucose initiates insulin secretion, which increases serum insulin concentrations that act on the skeletal muscle insulin receptors in order to stimulate GLUT4 translocation from the cytoplasm to the cell membrane to take glucose out of the blood, and promoting storage as glycogen, thereby lowering serum glucose levels (Watson & Pessin, 2001). When insulin resistance occurs, as in DM2, the insulin signaling cascade fails to properly initiate normal cellular responses due to the presence of excess lipids in the skeletal muscle. Under these circumstances GLUT4 proteins fail to translocate, dock and fuse into the plasma

membrane thus preventing glucose uptake from the blood (Kim et al, 2000; Dietz et al, 2002). This contributes to the excess glucose accumulation in the serum and partially explains the basis for hyperglycemia associated with type 2 diabetes mellitus.

In vivo NMR studies have shown glucose transport across the plasma membrane by GLUT4 to be a key regulatory point in insulin-mediated muscle glycogen synthesis, a key step in glucose uptake that limits glycogen synthesis (Rothman, 2001). Because increases in plasma free fatty acid concentrations can reduce GLUT4 trafficking to the plasma membrane, thereby inhibiting glucose transport, this suggests that defects in fatty acid metabolism do indeed have a key role in the pathogenesis of DM2 by interfering with insulin activation of glucose transport into the myocyte (Peterson & Shulman, 2002).

Fasting and Nonfasting *In Vivo* Metabolism

While McGarry's theory has been well-researched and has gained support from the diabetes research community (Peterson & Shulman, 2002), most of the studies concerning fatty acid metabolism and glucose regulation have been done using animals in the fasted state (McGarry, 2001). Because

fasting has profound and persistent effects on metabolism and the hormones that regulate substrate-utilization, it is difficult to envision how results obtained from fasted animals reflect the natural metabolic state of animals that are obese due to chronic over-eating (Cettour-Rose et al, 2002). In our own lab, skeletal muscle from high-fat (HF) fed rats was found to be able to oxidize IMCL while maintaining serum glycemia at the level of the lean control-fed animals (Jun & Fehn, 2003). Two key differences in our study and those of other investigators were the use of non-fasted animals and the concurrent analysis of both the extramyocellular lipids (EMCL) surrounding the myocyte and the IMCL deposits. IMCL in comparison to the EMCL revealed lipid influx into the IMCL lipid stores. However, the results from similar studies should be interpreted with caution and their methodology closely examined because many of these studies have measured total muscle lipids from whole muscle homogenates rather than distinguishing EMCL and IMCL by morphological analysis as was done in our lab. In light of this unexpected finding that the EMCL decreased in direct proportion to rising IMCL levels, we concluded that it is more likely that the skeletal muscle may, in fact,

preferentially utilize lipids, rather than being unable to use glucose as a substrate. This finding was also confirmed through indirect calorimetry which showed that the animals were utilizing lipids as their metabolic substrate (Jun & Fehn, 2003; Kelley & Mandarino, 2000). The shift to lipid oxidation is proposed to reduce glucose utilization, thereby contributing to the elevation of serum glucose concentrations that result from decreased disposal. In a subsequent study we discovered that plasma membrane GLUT4 concentrations in the skeletal muscle of the non-fasted obese animals were proportionate to the plasma insulin levels (Jun & Fehn, 2003) indicating the persistence of insulin responsiveness. Our results stand in sharp contrast to the dominant paradigm that insulin resistance due to IMCL is the main pathology underlying type 2 diabetes (McGarry, 2001; Jun et al, 2004). The one variable that has remained different in our studies in comparison to others is the use of non-fasted subjects.

Nutrient Regulation by Leptin and Thyroid Hormone

A complete picture of the physiology that underlies type 2 diabetes involves essentially every system in the body. The main theme throughout the study of this disease

is the dysfunctional trafficking of nutrients between various synthetic and storage compartments such as the liver, adipose tissues, and the skeletal muscle (Figure 2) (Lewis et al, 2002; Fehn et al, 2002). These compartments are intricately regulated principally by the peripheral sympathetic nervous system pathways that are under central nervous system control at the hypothalamus (Flier et al, 2000). The main control center for appetite and satiety, the hypothalamus, is a key regulator of feeding and metabolism. It gains information about peripheral food stores via nutrient-sensing pathways that involve hormonal signaling through the leptin and thyroid hormone pathways (Figure 3) (Fehn et al, 2002; Flier et al, 2000).

Leptin is a hormone secreted in proportion to the amount of fat stored in adipocytes and it regulates feeding by acting as a nutrient level indicator for the brain. While leptin has been thought of mainly as a satiety factor or the stop signal for feeding, it is more likely a "fuel gauge," signaling the hypothalamus of lowered nutritional status. Leptin increases metabolism by stimulating hypothalamic activity that is carried out through the peripheral sympathetic neurons, particularly in the skeletal muscle (McGarry, 2001; Flier et al, 2000). Leptin

works closely with the thyroid hormone 3,5,3'-triiodothyronine (T_3) in order to control the desire to eat and to signal satiety stop when the individual has consumed sufficient quantities of food (Castello et al, 1994; Diano et al, 1998). Because the hypothalamic responses to leptin are conveyed to peripheral tissues through sympathetic neurons, T_3 can increase peripheral responsiveness to leptin-regulated processes by enhancing expression of β -adrenergic receptors in target tissues like the liver, adipose tissues and the skeletal muscle (Chu et al, 1985; McGarry, 2001; Peterson & Shulman, 2002). Thus, there is a clear link between the regulation of metabolic responses by leptin and their modulation by thyroid hormone (Fehn et al, 2001; Fehn et al, 2002). In as much as the serum concentration of thyroid hormone is altered by fasting, this is but one source of potential error in interpreting studies of metabolic regulation by leptin using subjects with restricted food intake.

At the level of target tissues, T_3 controls additional response elements which must be taken into consideration when evaluating metabolic regulation. In addition to being responsive to insulin, skeletal muscle GLUT4 protein synthesis as well as trafficking are also stimulated by T_3 .

Thyroid hormone action on gene expression is mediated primarily at the level of gene transcription and is most prominent in the red muscle fiber types although thyroid hormone stimulates GLUT4 expression in both red and white skeletal muscle fiber types (Castello et al, 1994). Studies by Torrance et al (1997) have demonstrated that administration of T_3 was highly effective in augmenting GLUT4 mRNA and protein expression in obese-insulin resistance rat skeletal muscle of animals that were induced into hypothyroidism prior to treatments. This work points out the potential for thyroid hormone mediated GLUT4 expression in insulin resistance when T_3 is replaced to correct a pathological hypothyroid state, but it failed to test whether these actions are in effect under the thyroidal status normally present in obese diabetic subjects.

The thyroid system is regulated at multiple levels, one or more of which might account for nutritional adaptation. First, thyrotropin-releasing hormone (TRH), a neuropeptide produced in the paraventricular nucleus (PVN) of the hypothalamus, controls the release of thyroid-stimulating hormone (TSH) from the pituitary. TSH acts on receptors on the thyroid to promote synthesis and release

of the inactive thyroid hormone T_4 which is then converted to T_3 in peripheral tissues like the liver and kidney (Figure 3) (Flier et al, 2000; Silva et al, 1982). T_3 is released post-prandially in order to increase metabolism and promote lipolysis (Wilcox et al, 1991; Hoppner & Seitz, 1989) and probably hepatic lipogenesis. In a lean individual, T_3 elicits skeletal muscle thermogenic activity through the induction of uncoupling proteins (UCPs) for non-shivering thermogenesis and stimulates GLUT4 trafficking to the skeletal muscle plasma membrane (Ribiero et al, 2001; Castello et al, 1994; Torrance et al, 1997). Our lab has established that T_3 reduces IMCL stores while raising fatty acid metabolism in the skeletal muscle (Fehn et al, 2002; Goodridge et al, 2003). This again contradicts findings in the majority of the current literature on diabetes research, in which defective skeletal muscle lipid oxidation is often cited as the root of insulin resistance (McGarry, 2001; Tremblay et al, 1995). The practice of fasting, therefore, becomes even more critical since the dramatic and persistent decline in circulating T_3 levels prior to treatment can affect parameters such as whole-body metabolism, especially glucose homeostasis and substrate utilization. The goal of

this thesis was to address the potentially large role that fasting could have in the obese, diabetic animal model and their insulin sensitivity.

Current models of obesity and the development of type 2 DM have proposed that if an individual overeats, that he/she can eventually become insensitive to the satiety signals that control appetite, thereby becoming obese and perpetuating the oversecretion of hormones like insulin. Such a dysregulation of nutrient intake can lead to excess lipid accumulation in peripheral tissues that eventually causes secondary defects in metabolism such as insulin resistance (McGarry, 2001; Tremblay et al, 2001; Hawkins et al, 2002). Similarly, chronic over-nutrition could promote excessive thyroxine deiodination leading to chronic elevations in serum T_3 . Alternatively, peripheral resistance to T_3 may arise just as it has been theorized that insulin resistance arises when tissues like the liver and skeletal muscle fail to respond to elevated serum insulin in the presence of excess fats (Boden et al, 2001, McGarry, 2001). Physiological systems often see the loss of regulation as a loss of the signal altogether. Such a sequence of events would lead to the accumulation of IMCL and the development of insulin resistance that could

progress and develop into overt type 2 diabetes (Chatterjee, 2001). These are potential sequela that warrant further attention if the normal progression toward type 2 diabetes is to be understood.

It is well-established that the serum concentrations of both insulin and thyroid hormone drop dramatically after an overnight fast in non-obese animals, but these responses have not been investigated simultaneously in the diet-induced obese-diabetic rat model (Byerley & Heber, 1996; Diano et al, 1998). An overnight fast is a practice that is standard in clinical and animal research studies. This method is known to cause a sharp drop in the level of T_3 , mainly due to the lack of conversion from T_4 to T_3 by Type I deiodinase (Byerley, 1996; Fehn et al, 2001). Since T_3 is an established stimulant of GLUT4 expression in the skeletal muscle, the interpretation of results from experiments that use fasted animals as their subjects may be compromised (Torrance et al, 1997). The proposed work sought to bridge this gap by characterizing the effects of fasting on serum concentrations of insulin and T_3 and GLUT4 trafficking in the skeletal muscle of high-fat diet induced insulin resistant diabetic rats.

Purpose of the Thesis

This thesis project will investigate the effects of fasting on the serum levels of two key regulatory hormones, insulin and thyroid hormone (T_3) and the effects of these hormones on the trafficking of GLUT4 in soleus muscle. From this it will be possible to characterize the ability of T_3 to independently stimulate GLUT4 trafficking in the diet-induced obese-diabetic rat model.

Pilot Study on Hormone Interactions

In considering potential experimental designs, a pilot project was done to determine whether T_3 alters plasma insulin concentration in the fasted state. T_3 administration during fasting restored plasma insulin concentrations back to the levels of the non-fasted group. In the group that was fasted and then given replacement T_3 , their serum insulin and T_3 values were (mean \pm SEM) 93.2 ± 26.1 μ IU/ml and 81.8 ± 15.8 ng/dl, respectively. The control animals were fed *ad libitum*. Their serum insulin and T_3 values were 83.8 ± 9.5 μ IU/ml and 86.4 ± 17.3 ng/dl, respectively. Fasting with T_3 replacement, therefore, could not be used as an experimental manipulation to study thyroid hormone-regulated skeletal muscle GLUT4 trafficking

in the presence of fasting concentrations of insulin (Appendix B).

Independent versus Joint Effects of Insulin and T_3

This study aims to evaluate the independent versus joint effects of insulin and T_3 on GLUT4 trafficking using a novel approach to *in vivo* metabolic manipulation. Wistar rats will be fed a high fat diet for 11 weeks to create insulin-resistant, diet-induced obese (DIO) animal models. After insulin resistance is confirmed, all animals will be given streptozotocin (STZ) to stop endogenous insulin secretion by the pancreatic beta cells, thereby allowing the administration of replacement doses of insulin to mimic serum insulin levels in animals that are either fasted overnight or fed *ad libitum* to modify endogenous T_3 concentrations. Exogenous insulin administration will be paired with normal serum T_3 concentrations in animals permitted to feed *ad libitum* and with animals deprived of food overnight to induce fasting concentrations of T_3 . This will provide four combinations of fed and fasting concentrations of insulin and T_3 from which to evaluate the interaction of these hormones on soleus muscle GLUT4

transporter trafficking in a model of diet-induced insulin resistance.

CHAPTER TWO

MATERIALS AND METHODS

Animals

Study 1

Male Sprague-Dawley rats (Harlan Labs) weighing 51-75 grams were housed in groups of 3-4 per cage and maintained at 21-25°C, 14:10 hours light:dark (light 0600-2000h). All animals were fed a high-fat rodent chow with 45% of the total kilocalories derived from fat (Research Diets, Inc.). Food was available *ad libitum* throughout the study.

Study 2

Male Wistar rats (Charles River) weighing 50-75 grams were housed in groups of 3-4 per cage and maintained at 21-25°C, 14:10 hours light:dark (light 0600-2000h). All animals were fed a high-fat rodent chow with 45% of the total kilocalories derived from fat (Research Diets, Inc.) until they reached 17-19 weeks of age. Food was available *ad libitum* until the experimental treatments began, when the animals were either fasted or non-fasted, as described below.

Prior to group assignment, each animal underwent an oral glucose tolerance test (OGTT) in order to confirm

impaired insulin sensitivity compared to non-diabetic animals. Animals were fasted for 16 hours and then given a 40% glucose solution (Sigma Biochemical Co.) via an oral gavage tube at a dose of 1g glucose/kg body weight. Blood samples were collected by heel-stick using Safe-T Pro Lancets (Roche Pharmaceuticals). Blood glucose values were measured using Comfort Curve test strips in an Accu-Chek glucose meter (Roche Pharmaceuticals) at 0, 30, 60, 90, and 120 minutes following the oral glucose load.

Experimental Treatments

Study 1. Diet-induced Obesity (DIO) and Insulin Resistance in Response to High Fat Feeding

Groups of 43 male SD and 32 male Wistar rats that were provided high fat diet (as described above) were subjected to OGTT to confirm insulin resistance. SD animals served as high fat fed DIO animals refractory to induction of insulin resistance and Wistar animals served as the DIO responsive model. The insulin resistant Wistar animals were then employed for subsequent studies, as described below.

Study 2. Interactive Effects of Insulin
and T₃ on Soleus Muscle GLUT4 Trafficking -
Ablation of Pancreatic Beta Cells
and Insulin Secretion by
Streptozotocin (STZ)

Wistar rats having confirmed insulin resistance were divided into respective treatment groups (Table 1). In order to eliminate endogenous insulin production, all animals were fasted overnight and then received 80 mg/kg body weight streptozotocin (STZ, Sigma Biochemical Co.). The solution was prepared immediately before injection (20 mg/ml STZ in 0.05 M citrate, 0.15 M NaCl, pH 4.5,). Injections were administered intraperitoneally (i.p.) between 0800-0900h to induce type 1 diabetes as previously described (Zhou, 2004). Starting on the day after STZ injections, animals were provided with a 10% glucose solution to prevent hypoglycemia resulting from attenuated pancreatic alpha cell glucagon secretion that results from insulin deficit (Zhou, 2004). Type 1 diabetes was confirmed on the second day after the STZ injections using blood from heel-sticks and the Accu-chek method, as described above. All animals were given 1 unit (U) of Humulin N insulin (Lilly Pharmaceuticals) subcutaneously into their lower backs in order to prevent diabetic

complications and loss of life due to hypoinsulinemia and severe hyperglycemia.

Groups 1 and 2 served as the DIO fasted groups in which serum T_3 was reduced to the normal fasting concentration which would be complemented by either a full replacement dose of insulin (2 U) or a partial insulin dose (1 U) of Humulin N (Lilly Pharmaceuticals). Groups 3 and 4 were provided food (not fasted) in order to maintain normal non-fasting serum T_3 concentrations while being provided either the full or partial replacement dose of Humulin N.

Tissue Collection

Animals were euthanized in a CO_2 chamber which rendered them anesthetized, unconscious, and led to ventilatory arrest prior to death. Upon cessation of ventilation but prior to cardiac arrest approximately 1.0 ml of blood was withdrawn via cardiac puncture using a 23 gauge needle and a 1 ml syringe (BD Medical Supplies). The blood was placed on ice for approximately 15 minutes to allow clotting and then centrifuged at 1,000g for 10 minutes in order to separate the serum from the whole blood cells. Serum samples were aliquoted into separate 1.5 ml eppendorf tubes and stored in liquid nitrogen prior to RIA. Subsequent

bilateral thoracotomy and cardiac excision ensured that the procedure was terminal. The entire soleus muscle from each leg was collected from each animal and frozen in liquid nitrogen for storage prior to assay.

In Vivo Metabolic Analyses

Prior to and during the treatments, *in vivo* metabolic rates of each animal were measured through indirect calorimetry 10 minute readings at 400 ml/min using a flow-through system (Ametek Model S-3A Oxygen Analyzer and Model CD-SA Carbon Dioxide Analyzer) in order to quantify the amount of oxygen consumed and the carbon dioxide released in respiratory gasses. These measurements were used to compute the respiratory quotient ($RQ = \text{ml CO}_2 \text{ released} / \text{ml O}_2 \text{ consumed}$) that reflects the type of fuel (i.e. fats, carbohydrates or proteins) that the animals were utilizing as their primary energy source (Table 2). Baseline RQ readings were measured prior to treatments and throughout the 11-13 weeks of high-fat feeding.

Serum Hormone and Glucose Assays

Terminal point data include serum thyroid hormone concentrations (T_3), serum concentrations of insulin, blood

glucose levels and GLUT4 protein concentrations in the soleus muscle. Serum concentrations of insulin and thyroid hormone (T_3) were determined by commercial radioimmunoassay (RIA) (MP Biomedicals). Serum glucose concentrations were determined by the glucose oxidase colorimetric method microassay (Sigma Biochemical Co.) in a 96-well microtiter plate read at 520 nm.

Soleus Muscle GLUT4 Quantification

Immediately following removal from liquid nitrogen, 0.10 g of soleus muscle was placed in 1 ml of ice-cold STM buffer (10mM NaCl, 50mM Tris HCl pH 8.0, and 10mM $MgCl_2$ in distilled water). The mixture was homogenized on ice at maximum speed (Con-Torque) for one minute using a ground glass pestle. The homogenates were centrifuged at 4°C at 14,000g for five minutes to separate cytoplasmic vesicles containing GLUT4 from the plasma membrane GLUT4 fraction. The supernatant (cytoplasmic vesicular GLUT4 fraction) was aliquoted into a fresh tube. The pellet (plasma membrane GLUT4 fraction) was resuspended in 1 ml of ice-cold STM and sonicated (Fisher Scientific Model 500 Sonic Dismembrator with Ultrasonic Converter Model CL4) at setting "5" for 30 seconds to remove the docked vesicles and fused GLUT4

proteins from the plasma membranes. The sonicated fraction was centrifuged at 14,000g for 5 minutes at 4°C to separate the GLUT4 from the vesicles and the membrane. The supernatant (now containing only the plasma membrane GLUT4 protein fraction) was aliquoted into a fresh 1.5 ml microcentrifuge tube and the pellet discarded. The protein concentration in each homogenate was determined using the Bradford Assay (BioRad). Samples for subsequent analysis were standardized to each contain 100 µg of total protein and brought to 1000 µl with STM. Bovine serum albumin (Fraction V, Pierce) was used to generate the standard curve.

To each sample containing 100µg of total protein, 5 µl of diluted anti-GLUT4 primary antibody (5 µl primary antibody in 45 µl STM) GLUT4 antibody (GLUT4 rabbit polyclonal IgG, Santa Cruz Biotech) were added and each sample was vortexed. The samples were incubated at 4°C overnight to allow binding of primary antibody to GLUT4 proteins. Then 20 µl Protein A-Agarose (Santa Cruz Biochemicals) were added to each sample and each sample was vortexed. The samples were incubated at 4°C overnight while rocking to allow binding of the Protein-A Agarose to the primary antibody. Samples were then centrifuged at 14,000 g for 5

minutes at 4°C. The supernatant was removed and the pellet rinsed in 1 ml of ice-cold 1 x PBS (0.137 M NaCl, 0.00268 M KCl, 0.00147 M KH₂PO₄, 0.00801 M Na₂HPO₄ · 7H₂O, pH 7.3, autoclaved). Samples were rinsed, vortexed and centrifuged a total of three times in PBS. After the final rinse, 50 µl of 1x Laemmli treatment buffer (0.0625M Tris HCl pH 6.8, 2% SDS, 10% Glycerol, 5% 2(β)-mercaptoethanol) was added to the isolated GLUT4 pellet. The samples were stored at 4°C prior to gel electrophoresis.

The proteins were denatured by boiling for five minutes in a water bath and 15 µl of each sample was loaded onto a 0.75mm thick, 12% single concentration Tris-Glycine gel for electrophoresis (Jules, Inc.). Additionally, 5 µl of Molecular Weight Marker (Santa Cruz Biochemicals) were loaded onto each gel containing the samples. The gels were run for 40 minutes at 35 mA per gel (Hoefer Scientific instruments Mighty Small SE260; EC Apparatus Corp. EC-400 Power Source) in Laemmli tank buffer (0.025 M Tris, pH 8.3, 0.192 M Glycine, 0.1% SDS) with a refrigerated water circulator to maintain the temperature at 4°C (Cole Palmer Model 1268-14).

The proteins were transferred (Hoefer Scientific Instruments Model TE42) onto nitrocellulose (NC) membrane

(Osmonics) at five amperes overnight in Towbin buffer (0.25 M Tris, 0.192 M Glycine, 20% methanol, pH 8.3). Success of the transfer was confirmed by staining the gels with Coomassie Brilliant Blue to ensure absence of any remaining protein on the gel. The NC was blocked for 60 minutes in Blotto (5% Carnation Dry Milk in Tris Buffered Saline - 20mM Tris, 500 mM NaCl, pH 7.5), and the second incubation was carried out overnight at room temperature with shaking in 20 μ l primary antibody (Santa Cruz Biotech, GLUT4 rabbit polyclonal IgG) diluted in 100 ml blotto. This was followed by three TBS rinses of five minutes each. Then the NC membrane was incubated overnight in 20 μ l secondary antibody (Goat anti-rabbit IgG-HRP, Santa Cruz) diluted in 100ml Blotto followed by three ten minute rinses in TBS/0.05% Tween. Lastly, the NC membrane was rinsed in TBS once for five minutes. Equal volumes of Western Blotting Luminol Reagents A and B (Santa Cruz Biochemical Co.) using 0.125ml (125 μ l) per cm² of NC was used to incubate the NC for one minute. The NC was exposed and the film (Fuji Super RX, 8 x 10 in) developed using a Mohr Enterprise Model VP-200-ST film developer. GLUT4 concentrations were determined using NIH Image J software to obtain uncalibrated optical densities (OD) of the bands visualized

using transmittance digital photograph images of the films (UMAX Astra 1220U scanner).

Data Analyses

Statistical analyses were performed using SigmaStat Software (Version 3.1). All data are expressed as mean±SEM. Strain weight gain data was analyzed using one-way ANOVA in order to compare between groups of animals of varying ages. The effects of feeding status and insulin on serum hormone and blood glucose levels, glucose tolerance, and PM GLUT4 concentrations were analyzed using two-way ANOVA. Tukey *post-hoc* analysis was used for multiple comparisons in order to see which groups differed. ANOVA tables of critical values are listed in Appendix C.

CHAPTER THREE

RESULTS

High-Fat Diet and Weight Gain

Growth curves for male Wistar and Sprague-Dawley rats fed high-fat chow are shown in Figure 4. In the first 28 days, the Wistar animals gained, on average, 305 g and approximately 20-46 g/week thereafter, with maximal body weights of 501.3 ± 9.2 g being attained at 78-82 days of age (DOA). In comparison, the Sprague-Dawley rats (SDs) that began the same diet at the same age required 145-159 days to attain similar maximal weights of just under 500 g. The Wistar rats showed an increased propensity for early weight gain by attaining higher body weights by 78-82 DOA in comparison with SDs at 145-159 DOA (501.3 ± 9.2 v. 482.9 ± 7.44 g, respectively; $P < 0.001$; Table 3).

In Vivo Metabolic Analyses

In vivo indirect calorimetry was performed on the Wistar animals prior to and on the day of the STZ injections in order to assess which type of fuel the animals were metabolizing (Figure 5). A progressive shift toward higher RQ values as high-fat feeding continued

indicates that the animals were increasing carbohydrate utilization at the expense of lipid oxidation, as is typical of obese individuals developing insulin resistance (Bray & Bouchard, 2004). A drop in the RQ value on the last set of readings corresponds to the day following an overnight fast and injection of STZ in the previous evening. It should be noted that the values are slightly above 1.0 probably due to moisture in the analyzers that skewed the gas concentrations slightly.

Oral Glucose Tolerance Tests

All Wistar animals were confirmed to be insulin-resistant after an OGTT demonstrated impaired glucose tolerance following a 16h fast (Figure 6). The blood glucose concentrations were increased by 60% at 60 minutes following an oral glucose gavage and they remained elevated at 90 and 120 minutes, indicating a lack of glucose uptake by normal target tissues. In comparison, the non-resistant Sprague-Dawley rats showed blood glucose restored to baseline within 90 minutes of administering the glucose challenge. The blood glucose levels in the Wistar rats were greater than those in the SD rats by approximately 11%, 14%, and 17% at 60, 90, and 120 minutes, respectively

($P < 0.05$; Table 4). The Sprague-Dawley strain, which was found to be non-resistant; experienced a full recovery at a faster rate than the Wistar strain. At 60 minutes after the oral glucose load the serum glucose values of Wistar rats were higher and this continued through 120 minutes indicating insulin resistance. It is interesting to note that the SD rats actually began the OGTTs with higher baseline fasting blood glucose levels at 0 minutes.

. Serum T_3 , Insulin and Glucose Levels

In the presence of full doses of insulin (hi groups), as would be present in fed animals, fasting caused a significant decrease in serum T_3 concentrations (Figure 7) ($p < 0.01$; Table 5). Interestingly, there was also a significant ($p < 0.05$) decrease in serum T_3 in the fed (y) animals, with lower levels seen in the animals given the low (lo) dose of insulin, indicating possible interaction of the two hormones (Table 3). However, this was not seen in the fasted animals. All animals given the lower dose of insulin exhibited significantly reduced serum T_3 levels, suggesting that insulin may play a role in stimulating peripheral deiodination rates that are responsible for T_3 production. Whether T_3 and insulin act as antagonists to

each other is also a possibility, since each hormone has opposite effects on nutrient metabolism, with T_3 promoting breakdown and insulin promoting storage.

Exogenous insulin administration resulted in higher serum insulin levels (Figure 8) ($P < 0.05$; Table 6) in those animals that received the full replacement dose of 2 IU and lower serum levels in the animals that received the low dose (1 IU). This verified that the treatments provided the animals with the proper high and low serum insulin concentrations that were specified in the treatment protocol (Figure 8).

The impact of the insulin- T_3 treatment combinations on serum glucose concentration is shown in Figure 9. In the presence of high serum insulin concentrations representing a nonfasting state, a mild decrease in serum glucose was observed. All animals exhibited hyperglycemia, as would be expected in the insulin-resistant, DIO model. However, the animals that received high insulin doses did exhibit some lowering of blood glucose levels when compared to the groups that received low insulin doses, although the difference was significant at a slightly lower confidence limit ($p = 0.087$; Table 7).

Plasma Membrane GLUT4 Concentrations

GLUT4 concentrations were determined by Western blot of immunoprecipitates from 100 μ g plasma membrane total protein from soleus muscle homogenates (Figure 10). Plasma membrane GLUT4 concentrations did not differ between the treatment groups (Figure 11). Although there were significant differences in serum T_3 and insulin levels due to the treatments, muscle GLUT4 response exhibited little change among the groups (Figure 11), which is consistent with insulin resistance (Table 8). The lack of response to another direct regulator of GLUT4 trafficking, T_3 , may also indicate T_3 resistance at the peripheral target level of the skeletal muscle in the DIO model.

CHAPTER FOUR

DISCUSSION

This study was the first to elucidate in the diet-induced obese (DIO), diabetic animal model the joint versus independent roles of two key hormones responsible for nutrient homeostasis, insulin and T_3 . While much of the research in type 2 diabetes has focused intently on the roles of insulin and intramyocellular lipids on the regulation of skeletal muscle glucose transporter protein GLUT4, the role of T_3 on glucose homeostasis has not been explored. It has been established that T_3 directly affects GLUT4 trafficking to the plasma membrane as well as nuclear events leading up to an increase in GLUT4 protein synthesis (Castello et al, 1994; Torrance et al, 1997). Fasting has been shown to drastically reduce peripheral deiodination of T_4 to T_3 , but this is not taken into account during many, if not all, of the studies done on animals or humans in the fasted state (Byerley & Heber, 1996). The current study has identified, in DIO rats, changes in serum concentrations of T_3 that are modified by feeding status as well as an insulin-dependence in T_3 synthesis that is present only in the nonfasting state (Figure 7). Additional

analyses demonstrated the impact of these hormones on metabolic and nutrient trafficking responses. These findings underscore the importance of performing studies under the correct feeding status when evaluating the metabolic defects associated with insulin resistance and diabetes.

In order to confirm insulin resistance and impaired glucose uptake in the DIO Wistar rats, oral glucose tolerance testing was used to compare Wistar rats after an 11-week HF diet to SD rats that had been on the same diet for several months. When the two strains underwent the OGTT (Figure 6), SDs showed no sign of impaired glucose tolerance, unlike the Wistar rats that showed marked impairment in glucose uptake with a prolonged recovery to baseline after 120 minutes. It is interesting to note that these two strains reacted very differently to the same diet, with the SDs remaining insulin-sensitive while the Wistar rats developed Metabolic Syndrome X, and in a much shorter time on the HF diet than the SDs. A rather startling discrepancy in the rate of weight gain between the two strains was also observed (Figure 4). Because there are notable differences in both the development of obesity and insulin resistance, usage of either strain for

nutritional studies should be very carefully considered. The differences in the genetic backgrounds of these two particular strains also raise important and interesting questions about the human population and an individual's propensity toward becoming obese; not everyone who becomes obese will become diabetic. Clearly, genetic factors should be taken into account when studying obesity and the development of the Metabolic Syndrome as well as type 2 diabetes.

Concurrent with the weight gain in Wistar rats was a shift in the nonfasting RQ values (Figure 5) indicating increased carbohydrate utilization and decreased lipid oxidation that typically accompanies the development of obesity and insulin resistance (Bray & Bouchard, 2004). Thus, HF feeding of Wistar rats induced a number of physiological conditions that mimic major metabolic defects that are observed in human obesity, insulin resistance, and type 2 diabetes mellitus and provided a model with which to address the impact of fasting on insulin- T_3 interactions in metabolic regulation.

The effects of fasting on whole-body metabolism were observed in the initial phases of the study, when the RQ values of the Wistar rats declined after a 12-hour fast

(Figure 5). Clearly, this is not a benign effect, showing a short-term shift from carbohydrate utilization as a fuel source toward more lipid oxidation which occurs as a result of glucose-sparing mechanisms that are initiated during fasting. This itself has a significant impact not only on metabolic substrate specificity in tissues, but also on the animals' ability to make such a change under states of malnourishment, such as type 2 diabetes. It supports current research advocating calorie-restrictive diets in order to induce weight loss and the clearance of excess lipid depots from insulin-sensitive peripheral tissues like the skeletal muscle, adipose depots and liver (Gazdag et al, 2000). Fasting animals, therefore, could significantly impact the findings from such studies and, therefore, these results should be interpreted with caution.

One of the main goals of this study was to understand how insulin and T_3 worked to regulate nutritional homeostasis, first by finding how insulin, in the presence or absence of food, regulated glucose metabolism. After establishing insulin resistance in the DIO model, STZ treatment eliminated endogenous insulin production and allowed for the manipulation of serum insulin concentrations independent of feeding and glycemic status.

Concurrently, serum T_3 concentrations (Figure 7) were manipulated through the normal fasting inhibition of peripheral deiodination of T_4 . Serum T_3 was elevated in the group that received the full replacement dose of insulin while being given food, which represented the control group for this study. Interestingly, the animals that were fed and then given replacement or low doses of insulin experienced a drop in serum T_3 levels. Insulin appears to play a role in modulating non-fasting serum T_3 concentration by regulating peripheral deiodination of T_4 to T_3 in the nonfasted state. It should be noted, however, that this insulin effect on T_3 levels was observed only in those groups that were being fed *ad libitum* throughout the study. This can be explained by the inhibition of 5'MDI that already occurs with fasting (Byerley & Heber, 1996), which would have masked any additional effect that insulin may have had on peripheral deiodination, as was observed in the fed animals. However, T_3 was reduced by almost 50% in all other remaining groups that were fasted the night before termination, including those receiving high doses of insulin, which demonstrates the insulin-independent effect of fasting on peripheral deiodination of T_4 to T_3 (Byerley & Heber, 1996). It appears that there is an interaction

between insulin and T₃ activity. In this experiment, fasting was shown to play an important role in the inhibition of T₃ synthesis while insulin played a secondary role in maintaining serum T₃ concentrations at normal physiological levels under non-fasting conditions.

Serum insulin levels were also consistent with the exogenous dosages that were given to each treatment group, with both of the groups that received the full replacement insulin doses exhibiting elevated levels of insulin in comparison to the animals that received the half-dose of insulin (Figure 8). Although the decrease in serum glucose levels in response to high insulin dose failed to reach a statistical significance at $P < 0.05$ (Figure 9), it was apparent that lower glucose levels occurred in the groups that received the full replacement dose of insulin ($p = 0.087$), and the elevated levels of glucose were seen in the groups that received the lower dose of insulin. All subjects remained hyperglycemic in spite of the high serum insulin levels and this is consistent with the DIO phenotype that exhibits excessive glucose production by the liver and impaired glucose uptake by the peripheral tissues as a result of insulin resistance (McGarry, 2001). Thus, the model performed according to expectations with respect

to glucose intolerance due to attenuated insulin responsiveness and provided the opportunity to investigate the impact of insulin and feeding status on one of the key glucose trafficking controls, skeletal muscle GLUT4 transporter regulation.

The entry of glucose into cells is dependent upon the insertion of glucose transport proteins into the plasma membrane and attenuated insulin-stimulated GLUT4 trafficking in skeletal muscle is considered a major contributor to the development of hyperglycemia in type 2 diabetes mellitus (Boden et al, 2001; Kelley et al, 2000; Peterson & Shulman, 2002). But despite reports demonstrating that T_3 has the ability to induce GLUT4 synthesis and trafficking in the skeletal muscle in normal, nondiabetic animals (Castello et al, 1994; Torrance et al, 1997), these responses have not been investigated in models of DIO and insulin resistance.

It has been clearly established that peripheral deiodination declines significantly and thereby reduces serum T_3 when animals are fasted, as evidenced through literature (Byerley et al, 1996; Diano et al, 1998; Wilcox et al, 1991) as well as the results of the current study. Whether T_3 acts upon GLUT4 trafficking synergistically with

or independently from insulin was addressed by quantifying soleus muscle plasma membrane GLUT4 under four insulin- T_3 concentration combinations. Despite treatment combinations that exposed the soleus to high concentrations of both insulin and T_3 , high concentrations of insulin with low concentrations of T_3 , and low concentrations of both hormones, there were no differences in plasma membrane GLUT4 concentration between the control and treatment groups. Unexpectedly, the groups that were fed *ad libitum* and received half-doses of insulin did not develop the intended high concentrations of T_3 with low insulin concentrations (Figure 7).

Since all of the subjects employed in this study were insulin resistant DIO animals, it is not surprising that little or no change in GLUT4 trafficking was observed in response to different insulin concentrations (Figure 11). However, the important finding here is that there appears to be no fasting or feeding effect on plasma membrane GLUT4 concentrations, this despite the substantial decreases in serum T_3 concentrations that accompany fasting.

The lack of any response to feeding or fasting raises the possibility of parallel T_3 resistance in these animals. They were unable to respond to varying doses of insulin, a

known and potent stimulator of GLUT4 trafficking, and they did not respond to changes in nutritional status as reflected through serum T_3 , a hormone that affects GLUT4 synthesis as well as trafficking (Torrance et al, 1997). Although the mechanism by which T_3 regulates GLUT4 trafficking is not yet clearly understood, this study suggests the potential for T_3 resistance in DIO animals that is similar to insulin resistance. A common subcellular transduction pathway may be activated by T_3 in the same manner that insulin activates its downstream signaling cascade, and excessive lipids could similarly be causing blunted GLUT4 trafficking response to T_3 . The animals that received the full replacement dose of insulin while feeding *ad libitum* had T_3 levels that were greater than any of the other treatment groups (Figure 7). Yet this response was not reflected in the plasma membrane GLUT4 concentrations, nor was it observed in the animals that received high doses of insulin while fasting (Figure 11).

As a nuclear-acting hormone, T_3 may act indirectly to increase GLUT4 trafficking through its permissive role of activating transcription factors that up-regulate insulin receptor (IR) biosynthesis. The increase in the number of IR would increase the number of ligand-receptor

interactions, thereby stimulating more GLUT4 vesicular translocation and docking into the plasma membrane. If the intracellular environment is overloaded with lipids, there is a strong possibility of compromised T_3 uptake and/or translocation into the nucleus.

Alternatively, the disconnection between feeding status and its effect on GLUT4 trafficking may reflect the typically slow action of the nuclear-acting T_3 hormone. Given that the animals were fed throughout the study and only fasted overnight (16h) prior to tissue collection, the T_3 -associated mechanisms may have persisted and therefore not impacted the short-term regulation of GLUT4 trafficking. This could suggest a role for T_3 in the long-term regulation of insulin-responsiveness rather than in short-term changes in feeding and nutritional status.

There is evidence from this study that insulin influences deiodination of T_4 to T_3 in fed animals (Figure 7). Deiodination controls the amount of T_3 that is present in the blood, and T_3 in turn controls the expression of GLUT4 proteins in the myocytes (Byerley & Heber, 1996). Therefore, it may be possible that while insulin, which fluctuates with nutritional states over short periods of time, acts as a short-term regulator of GLUT4 trafficking,

T₃ acts as a long-term set point in GLUT4 synthesis and their availability within the cell.

In addition to affecting many of the same parameters as insulin, T₃ often affects them in opposite ways, for example, by promoting catabolism and release of stored nutrients rather than their synthesis and storage. It is apparent that insulin and T₃ may function variably as synergists and antagonists depending upon the target tissue and specific response. Also, like insulin, serum T₃ levels rise in response to feeding and decline in lower nutritional states, so it is reasonable to hypothesize that in the same manner that chronic hyperinsulinemia induces insulin resistance, continuously elevated T₃ levels may lead to peripheral and/or central resistance to T₃. One can speculate that this could then lead to a hypothyroid phenotype which would include decreased beta oxidation, increased gluconeogenesis, and attenuated thermogenesis, all of which would lead to insulin resistance, a hallmark of Metabolic Syndrome X.

T₃ has antagonizing effects on many of the same metabolic processes as insulin, so this may also play a large role in the dysfunctional nutrient homeostasis that is seen in obese individuals (Wilcox et al, 1991). Even

though T_3 has been shown to play an active role in GLUT4 protein synthesis and trafficking, its importance in the study of obesity and type 2 diabetes has been put aside for many years. T_3 affects virtually every process in nutrient homeostasis including lipid metabolism and most significantly, glucose homeostasis. Since fluctuations in T_3 can occur from relatively minor changes in nutritional status, i.e. an overnight fast, a practice that is standard in nutrition studies, T_3 deserves a closer look for its ability as a potent nuclear transcriptional activator as well as its direct effects on peripheral tissue metabolic processes.

This thesis represents a unique approach to studying the *in vivo* regulation of glucose homeostasis in the insulin-resistant, DIO animal model. The question of how insulin and T_3 jointly or independently regulate GLUT4 glucose transporter trafficking has been addressed for the first time in this study and the union of these hormonal actions into a model has been proposed for the short- and long-term regulation of GLUT4 transporters. Further, the profound impact on metabolic substrate selection of fasting subjects prior to collecting and interpreting data has been demonstrated. Studies into the nuclear transcriptional and

protein synthetic events that follow fasting-induced T_3 decline have yet to be undertaken in this obese, diabetic animal model and these will certainly offer an avenue of discovery for a more comprehensive understanding of this critical process in metabolic regulation.

Finally, unexpected differences in metabolic responses between the two genetic strains of experimental animals, Wistar and Sprague-Dawley, were reflective of some cases that are currently seen with obese human populations, when one overweight person ultimately develops type 2 diabetes while others do not ever develop any of its symptoms. Therefore, the choice of animal strains in specific studies should be carefully considered and additional studies designed to explore the genetic and physiological differences between these two models with the hope of better understanding the basis for metabolic variances in humans.

Future studies in the onset of the nuclear transcriptional and protein synthetic events that follow T_3 decline after an overnight fast has yet to be studied in the obese, diabetic animal and is certainly an avenue that should be further explored.

APPENDIX A

FIGURES

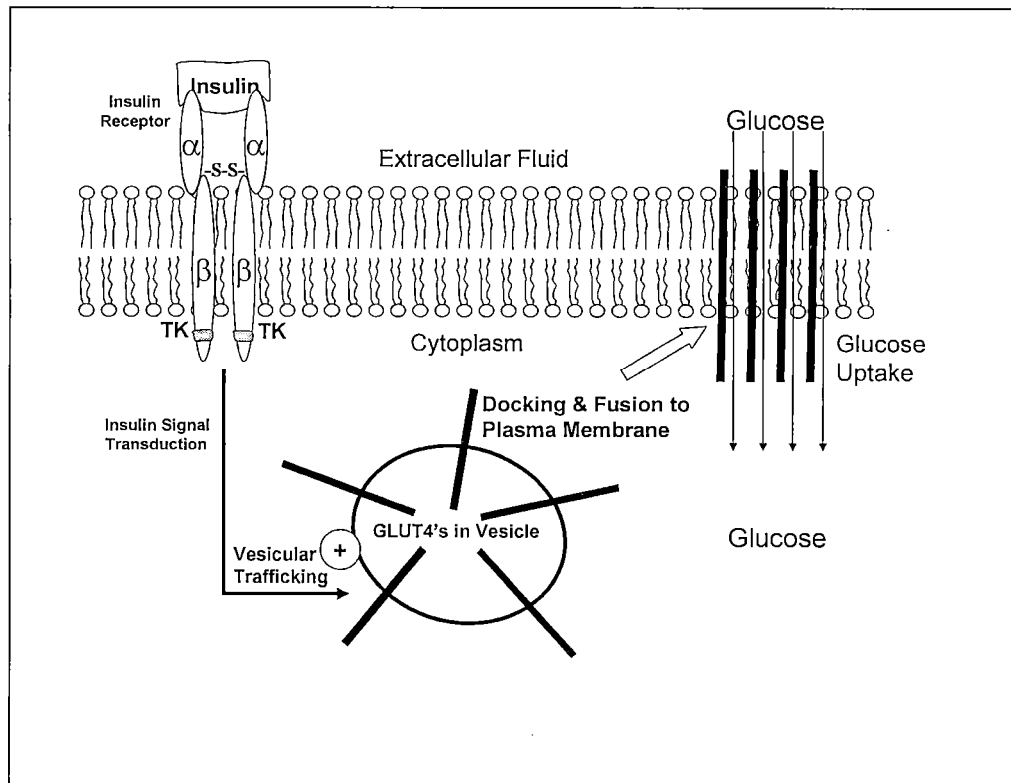


Figure 1. Insulin-Stimulated GLUT4 Trafficking. High serum glucose induces insulin secretion, which stimulates GLUT4 translocation from the cytoplasm to the cell membrane to enable glucose uptake from the blood and activation of enzymes to affect subsequent storage as glycogen, thereby lowering serum glucose levels (Diagram was reproduced with permission from Richard Fehn).

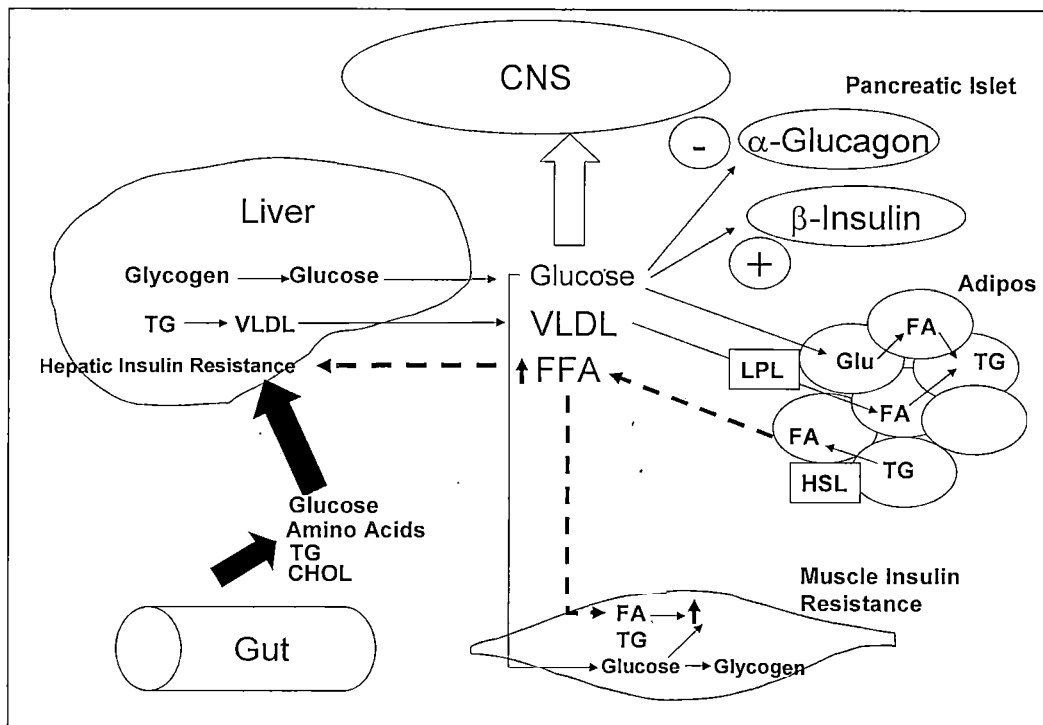


Figure 2. Dysfunctional Nutrient Trafficking in Type 2 Diabetes. Increased caloric intake and reduced energy expenditure leads to an accumulation of TGs in many tissues, especially adipose tissue, which causes FFA spillover to non-adipose tissue (dashed lines indicate major sites of dysfunctional trafficking). The liver, pancreas, and especially the skeletal muscle accumulate excess fats and eventually develop the insulin resistance characteristic of type 2 diabetes (Diagram was reproduced with permission from Richard Fehn).

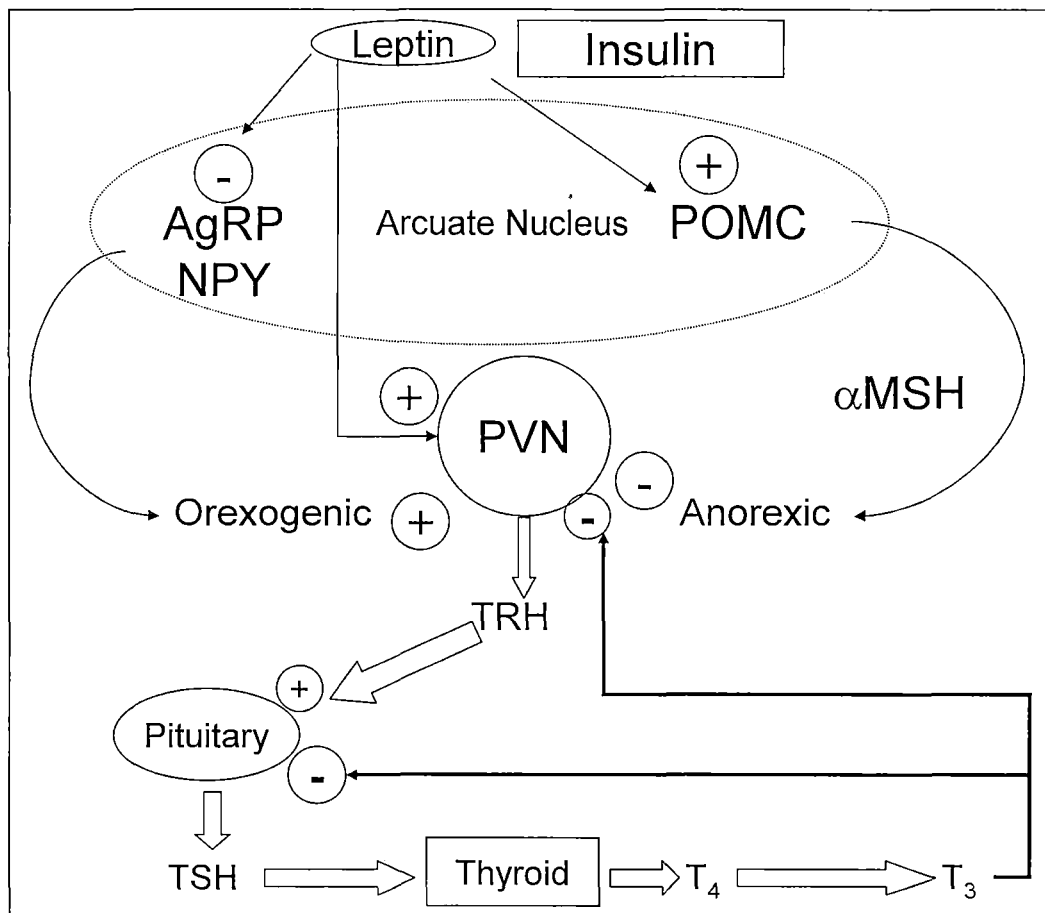


Figure 3. Hypothalamic Regulation of Metabolism. The hypothalamus is the main feedback center for the leptin signaling pathway through the arcuate nucleus. A sufficient level of leptin is needed to stimulate the paraventricular nucleus (PVN) to secrete thyrotropin-releasing hormone (TRH). TRH acts on the pituitary to stimulate thyroid-stimulating hormone (TSH). TSH then acts on the thyroid to promote the synthesis and release of thyroid hormones T_4 and T_3 . T_3 is the inhibitory feedback signal to the pituitary thyrotropes and PVN which stops TSH secretion (Diagram was reproduced with permission from Richard Fehn).

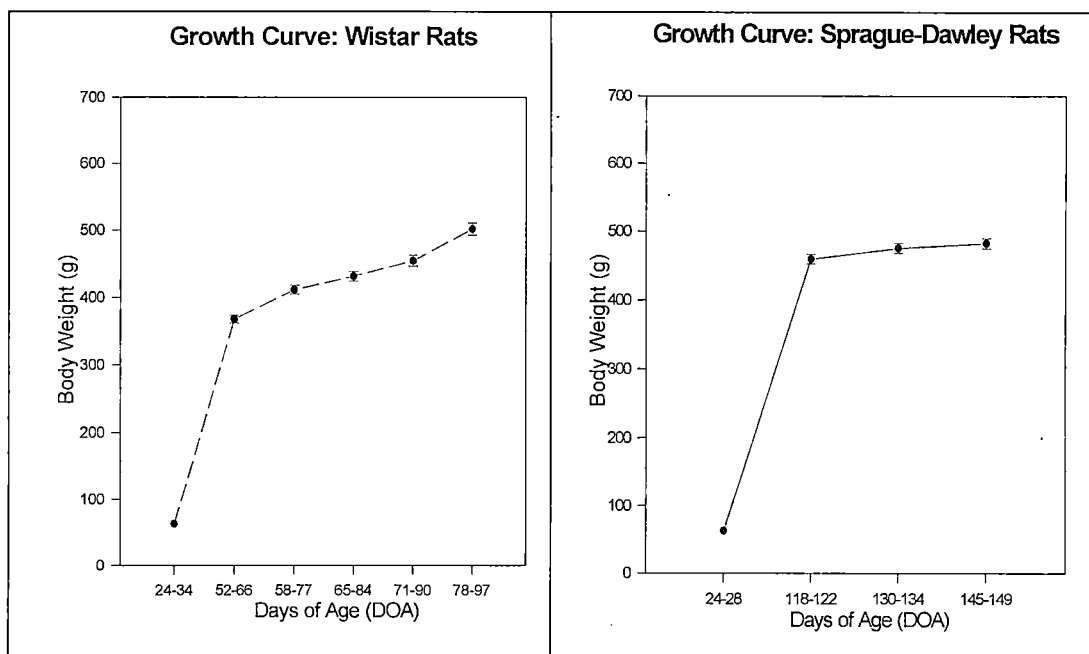


Figure 4. Wistar and Sprague-Dawley Growth Curves. Growth curves while feeding on a 45% kcal by fat diet over a period of a) 11.7 weeks for Wistar rats and b) 145-159 days for Sprague-Dawley rats. In spite of a longer period of feeding on the HF chow, the SD rats at 145-159 days of age remained significantly smaller than Wistar rats at 78-97 days of age ($p < 0.001$). Values are mean \pm SEM, $n=28-32$.

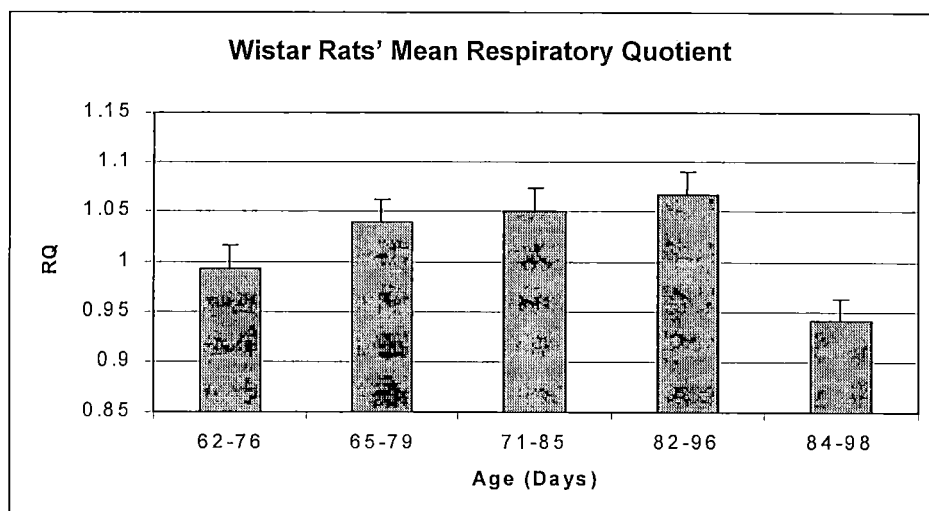


Figure 5. The RQ of the Wistar rats increased progressively with the development of obesity as resistance developed and lipid metabolism decreased. A drop in RQ value indicating increased lipid catabolism was observed following an overnight fast and STZ injection (84-98 Days). Values are mean \pm SEM, n=32.

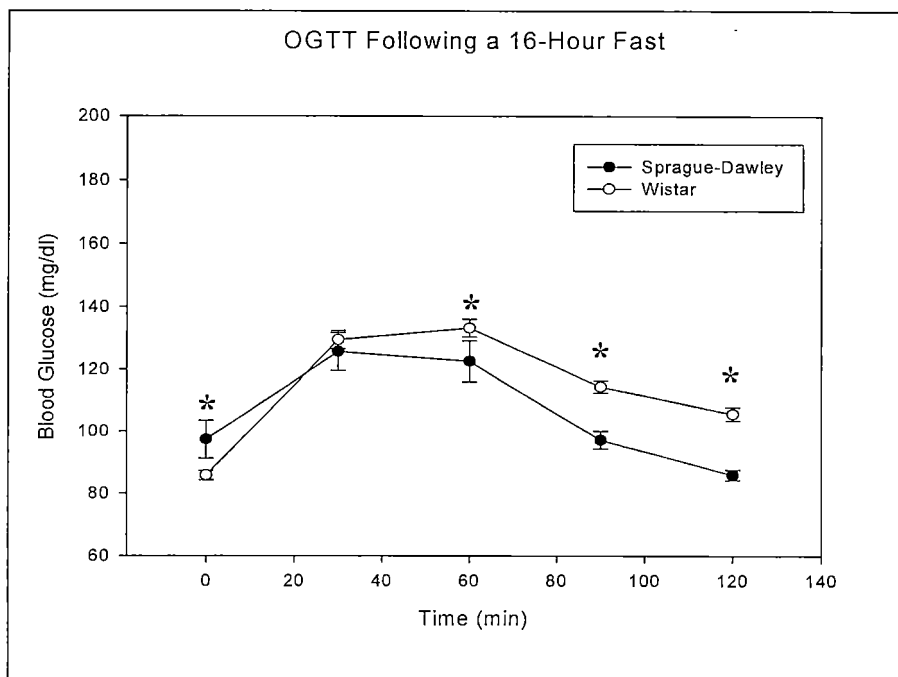


Figure 6. Wistar rats showed significantly impaired glucose tolerance in comparison to nondiabetic SDs despite higher baseline fasting glucose levels in the SDs ($p < 0.05$). Values are mean \pm SEM, $n = 28-32$.

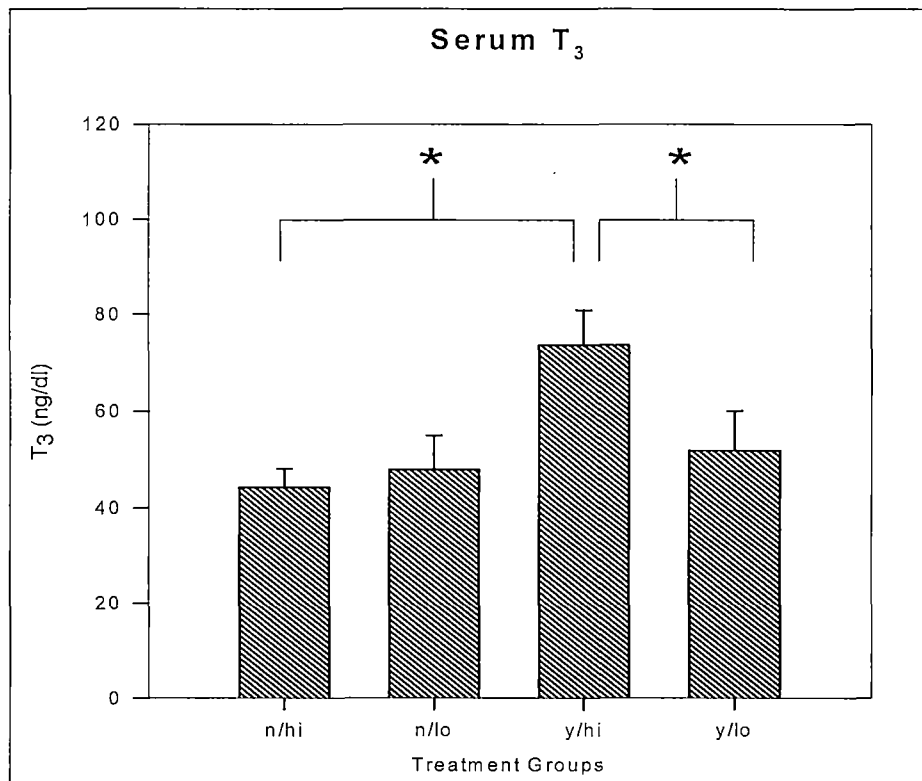


Figure 7. Serum T₃ concentrations were maintained at nonfasting levels in fed animals that received the full replacement dose of insulin (y/hi). The serum T₃ levels dropped significantly ($p < 0.01$) after fasting in the animals that received the high doses of insulin (n/hi). This fasting effect was not observed in the group that received lower doses of insulin (n/lo). The fed animals receiving low insulin (y/lo) also had a drop in T₃ ($p < 0.05$). All values are mean \pm SEM, $n = 7-8$.

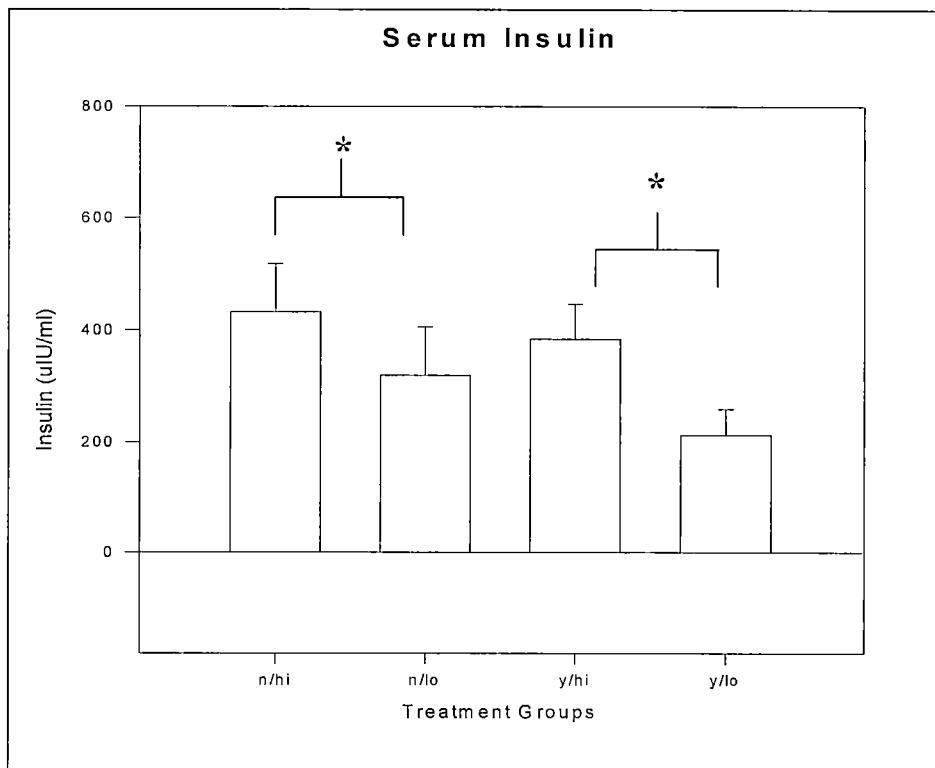


Figure 8. Exogenous insulin replacement with high doses resulted in the higher serum insulin levels seen in the n/hi and the y/hi groups. The animals that received half doses, n/lo and y/lo showed significantly lowered serum insulin in comparison to the hi groups ($p < 0.05$). Values are mean \pm SEM, $n = 7-8$.

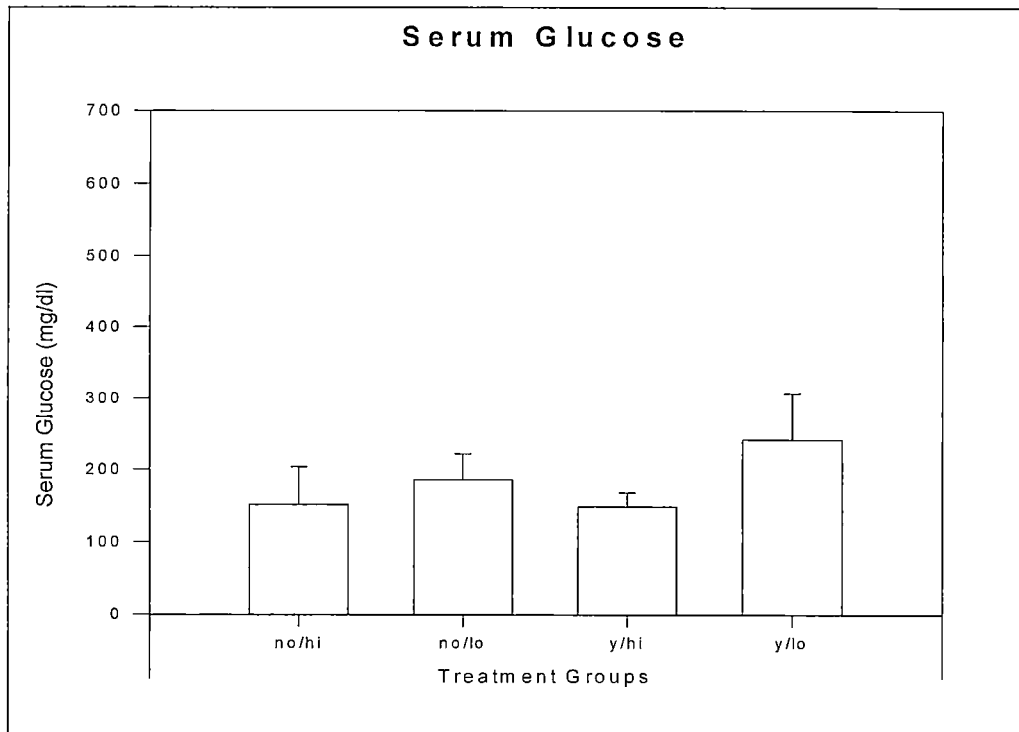


Figure 9. Serum glucose levels were lowered slightly in the high insulin dose groups in comparison to the low insulin dosages, although only to a statistical significance level of ($p=0.087$). This lack of response indicates insulin resistance in the DIO model. Values are mean \pm SEM, $n=7-8$.

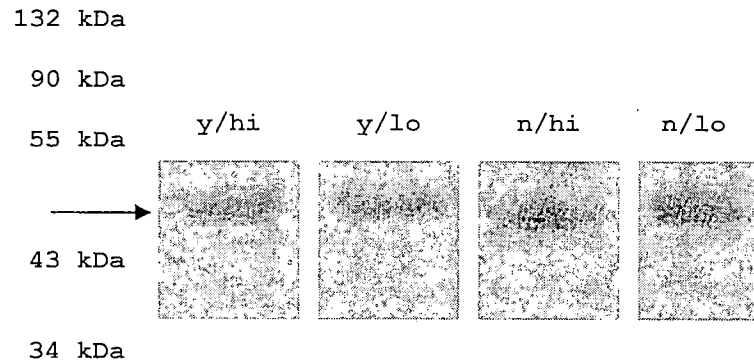


Figure 10. Representative GLUT4 Protein Bands from Each Treatment Group. Representative Western blot of GLUT4 protein bands were obtained from 100µg of soleus muscle plasma membrane total protein. A MW ladder is represented in the first column. Treatment groups that were fed (y) or fasted (n) and those receiving replacement (hi) or low (lo) insulin doses are shown in columns 2-5. GLUT4 proteins migrate at a position indicating a MW of approximately 49.5 kDa (Arrow) (Ryder et al, 1999). Values are mean±SEM, n=7-8.

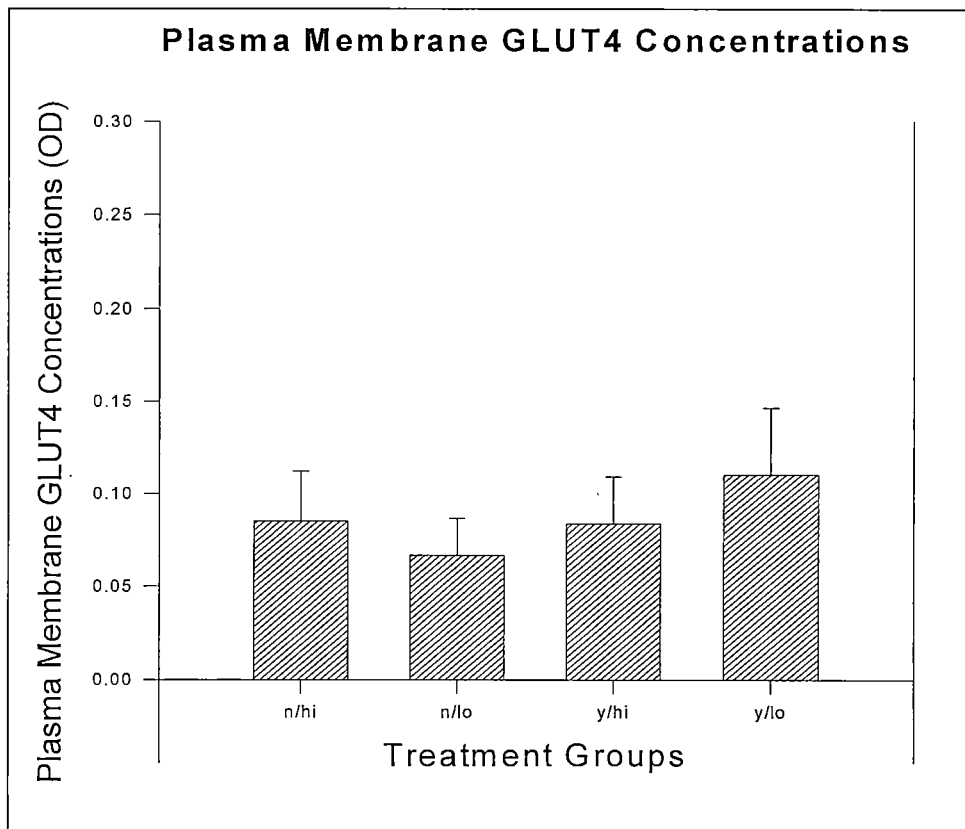


Figure 11. Plasma membrane GLUT4 content was compared using densitometric scans of Western blots. None of the groups showed significant changes in GLUT4 concentrations in response to changing concentrations of insulin and T_3 indicating resistance to both hormones with respect to regulation of GLUT4 trafficking. Values are mean \pm SEM, n=7-8.

APPENDIX B
PILOT STUDY DATA

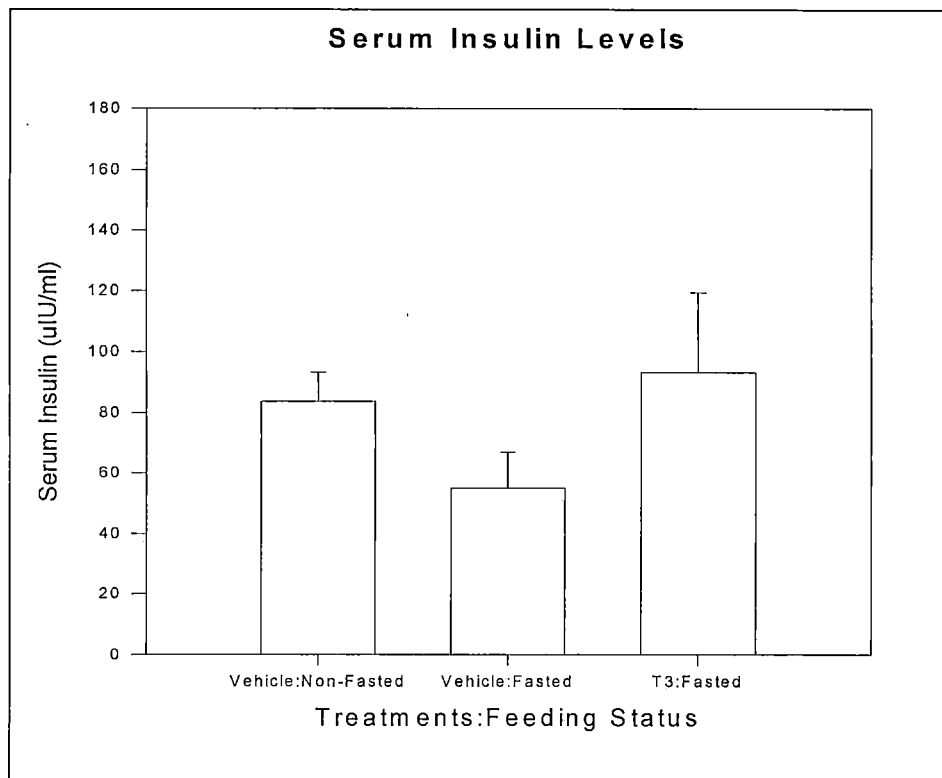


Figure 1. The control group (Vehicle:Non-fasted) was given saline vehicle and fed *ad libitum* throughout the study. Another group was fasted overnight (12 hours) and received a replacement dose of T_3 in order to compensate for the decline in T_3 that results due to decreased peripheral T_4 to T_3 deiodination during starvation states like fasting (T_3 :Fasted). The serum insulin levels in the T_3 :Fasted group appeared to have been restored to that of the control group. However, these results did not reach statistical significance, probably due to the small sample numbers per group ($n = 5$) which resulted in high within-group variability. In spite of the lack of statistically significant differences between the control animals (Vehicle:Non-Fasted) and the fasted animals given replacement T_3 (T_3 :Fasted), it did appear that T_3 may be regulated by insulin to some degree. It was concluded that relying solely on fasting with exogenous T_3 replacement in order to control insulin and T_3 levels independently of each other would not be a sound approach. Values are in mean \pm SEM, $n=5$.

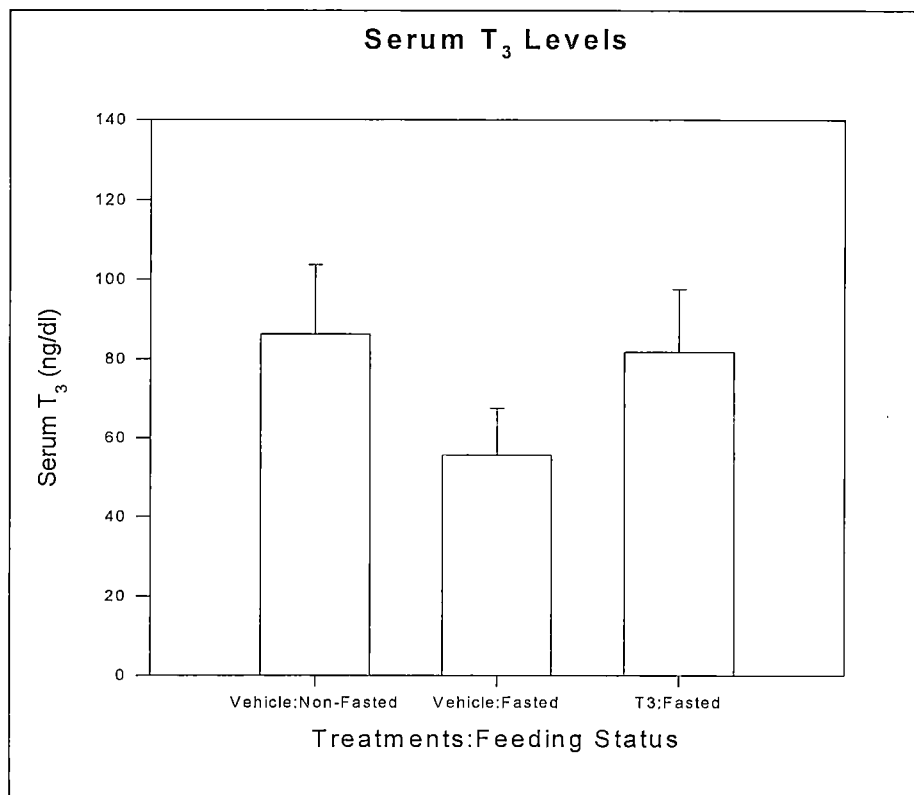


Figure 2. The serum T₃ levels paralleled the insulin concentrations and supported the possibility of interaction between insulin and T₃. Values are in mean±SEM, n=5.

APPENDIX C

TABLES

Table 1. Treatment Protocol for Study 2.

Treatment Groups	n	Food	Insulin	Symbols
1	7	No	Low Dose of Insulin	n/lo
2	8	No	Replacement Dose of Insulin	n/hi
3	7	Yes	Low Dose of Insulin	y/lo
4	8	Yes	Replacement Dose of Insulin	y/hi

Table 2. Respiratory quotient values under varying substrate utilization conditions.

Substrate	RQ
Carbohydrates	1.00
Proteins	0.74
Lipids	0.71

Table 3. One-Way ANOVA - Comparing Body Weights Between Two Strains at Different Ages

Source of Variation	DF	SS	MS	F	P
Between Groups	9	7874325.324	874925.036	617.330	<0.001
Residual	312	442188.956	1417.272		
Total	321	8316514.280			

Table 4. Two-Way ANOVA - Testing the Effects of Time and Strain on Glucose Tolerance

Source of Variation	DF	SS	MS	F	P
Time	4	109669.522	27417.380	133.392	<0.001
Strain	1	23792.109	23792.109	115.754	<0.001
Time x Strain	4	16088.188	4022.047	19.568	<0.001
Residual	350	71938.925	205.540		
Total	359	222011.900	618.418		

Table 5. Two-Way ANOVA - Testing the Effects of Feeding and Insulin on Serum T₃ Levels

Source of Variation	DF	SS	MS	F	P
Feeding Status	1	2063.495	2063.495	5.699	0.025
Insulin	1	612.142	612.142	1.691	0.205
Feeding Status x Insulin	1	1216.011	1216.011	3.358	0.078
Residual	26	9414.200	362.085		
Total	29	13108.339	452.012		

Table 6. Two-Way ANOVA - Testing the Effects of Feeding and Insulin on Serum Insulin Levels

Source of Variation	DF	SS	MS	F	P
Fed	1	140573.461	140573.461	4.502	0.039
Insulin	1	344880.339	344880.339	11.046	0.002
Fed x Insulin	1	271.288	271.288	0.00869	0.926
Residual	48	1498668.953	31222.270		
Total	51	1939817.903	38035.645		

Table 7. Two-Way ANOVA - Testing the Effects of Feeding and Insulin on Serum Glucose Levels

Source of Variation	DF	SS	MS	F	P
Feeding	1	8119.737	8119.737	0.526	0.472
Insulin	1	47203.213	47203.213	3.057	0.087
Feeding x Insulin	1	9890.832	9890.832	0.640	0.427
Residual	49	756726.444	15443.397		
Total	52	832640.707	16012.321		

Table 8. Two-Way ANOVA - Testing the Effects of Feeding Status and Insulin on Plasma Membrane GLUT4 Concentrations

Source of Variation	DF	SS	MS	F	P
Feeding Status	1	0.00359	0.00359	0.575	0.454
Insulin	1	0.000132	0.000132	0.0212	0.885
Feeding Status x Insulin	1	0.00407	0.00407	0.653	0.426
Residual	28	0.175	0.00624		
Total	31	0.183	0.00589		

LIST OF REFERENCES

1. Boden, G., Lebed, B., Schatz, M., Homko, C., & Lemieux (2001). Effects of acute changes in plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects. *Diabetes*, 50, 1612-1617.
2. Byerley, L.O. and Heber, D. (1996). Metabolic effects of Triiodothyronine replacement during fasting in obese subjects. *Journal of Clinical Endocrinology and Metabolism*, 81, 968-976.
3. Castello, A., Rodriguez-Manzanegue, J.C., Camps, M., Perez-Castillo, A., Testar, X., Palacin, M., Santos, A and Zorano, A. (1994). Perinatal hypothyroidism impairs normal transition of GLUT4 and GLUT1 glucose transporters from fetal to neonatal levels in heart and brown adipose tissue. Evidence for tissue-specific regulation of GLUT4 expression by thyroid hormone. *The Journal of Biological Chemistry*, 269, 5905-5912.
4. Cettour-Rose, W., Burger, A.G., Meier, C.A., Visser, T.J. and Rohner-Jeanrenaud, F. (2002). Central stimulatory effect of leptin on T₃ production is mediated by brown adipose tissue. *American Journal of Endocrinology and Metabolism*, 283, E980-E987.

5. Chatterjee, V.K. (2001). Resistance to thyroid hormone and peroxisome-proliferator-activated receptor gamma resistance. *Biochemical Society Trans*, 29, 227-31.
6. Chu, D.T., Shikama, H., Khatra, B.S. & Exton, J.H. (1985). Effects of altered thyroid status on β -adrenergic actions on skeletal muscle glycogen metabolism. *The Journal of Biological Chemistry*, 260, 9994-10000.
7. D'Alessandro, M.E., Chicco, A., Karabatas, L. and Lombardo, Y.B. (2000). Role of skeletal muscle on impaired insulin sensitivity in rats fed a sucrose-rich diet: Effect of moderate levels of dietary fish oil. *Journal of Nutritional Biochemistry*, 11, 273-280.
8. Diano, S.A., Naftolin, F., Goglia, F. and Horvath, T.L. (1998) Fasting-induced increase in Type II iodothyronine deiodinase activity and messenger ribonucleic acid levels is not reversed by thyroxine in the rat hypothalamus. *Endocrinology*, 139, 2879-2884.
9. Dietze, D., Koenen, M., Kohrig, K., Horikoshi, H., Hauner, H. & Eckel, J. (2002). Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. *Diabetes*, 51, 2769-2376.

10. Fehn, R., C. Clark, B. Underhill, R. Moussa , J. Donkor, 2001 A Role for Leptin in Regulating Peripheral Thyroxine Monodeiodination. *FASEB J.* 15(4):A597 480.6.
11. Fehn, R., R. Moussa, and J. Donkor. 2002 Dyslipidemia in Obese Mice: Regulation by Leptin and Thyroid Hormone Following Peripheral Sympathetic Neural Ablation. *FASEB J.* 16(4):A399, 361.4.
12. Fehn, R., R. Moussa, and J. Donkor (2002) Leptin and Thyroid Hormone in Metabolic Substrate Selection and Trafficking following Sympathetic Ablation. *Diabetes* 51(2):A581, 2407.
13. Flier, J.S., Harris, M. and Hollenberg, A.N. (2000). Leptin, nutrition and the thyroid: The why, the wherefore, and the wiring. *The Journal of Clinical Investigation*, 105, 859-861.
14. Gazdag, A.C., Wetter, T.J., Davidson, R.T., Robinson, K.A., Buse, M.G., Yee, A.J., Turcotte, L.P. & Cartee, G.D. (2000). Lower calorie intake enhances muscle insulin resistance and hexosamine levels. *The American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 278, 504-512.
15. Goodridge, A.G., Garay, A. & Silpananta, P. (1974). Regulation of lipogenesis and the total activities of

lipogenic enzymes in a primary culture of hepatocytes from prenatal and early postnatal chicks. *The Journal of Biological Chemistry*, 249, 1469-1475.

16. Goran, M.I., Ball, G. and Cruz, M.L. (2003).

Cardiovascular Endocrinology 2: Obesity and risk of type 2 diabetes and cardiovascular disease in children and adolescents. *The Journal of Clinical Endocrinology and Metabolism*, 88, 1417-1427.

17. Hawkins, M., Gabriely, H., Wozniak, R., Reddy, K., Rossetti, L. & Shamon, H. (2002). Glycemic control determines hepatic and peripheral glucose effectiveness in Type 2 diabetic subjects. *Diabetes*, 51, 2179-2189.

18. Hoppner, W. & Seitz, H.J. (1989). Effect of thyroid hormones on glucokinase gene transcription in rat liver. *The Journal of Biological Chemistry*, 264, 20643-20647.

19. Jun, L., Anderson, E., An, J., Clark, C., Newgard, C., and Fehn, R. (2004). Persistent insulin-stimulated GLUT4 trafficking despite high-fat induced intramyocellular lipids. *FASEB J.*, A7397.

20. Jun, L. and Fehn, R. (2003). Short-term increase in intramyocellular lipid content supports enhanced lipid oxidation without compromising serum glycemic status. *FASEB J.* 17(4): A275, 177.5. A8188.

21. Kelley, D.E. and Mandarino, L.J. (2000). Fuel selection in human skeletal muscle in insulin resistance. A reexamination. *Diabetes*, 49, 677-683.
22. Kim, Y.B., Nikoulina, S.E., Ciaraldi, T., Henry, R.R. & Kahn, B.B. (1999). Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *The Journal of Clinical Investigation*, 104, 733-741.
23. Kuhlmann, J., Newmann-Haefelin, C., Ulrich, B., Kalisch, J., Juretschke, H.P., Stein, M., Kleinschmidt, E., Kramer, W. & Herling, A.W. (2003). Intramyocellular lipid and insulin resistance: A longitudinal *in vivo* ^1H -spectroscopic study in Zucker diabetic fatty rats. *Diabetes*, 52, 138-144.
24. Laybutt, D.R., Schmitz-Peiffer, C., Saha, A.K., Ruderman, N.B., Biden, T.J. & Kraegen, E.W. (1999). Muscle lipid accumulation and protein kinase C activation in the insulin-resistant chronically glucose-infused rat. *Journal of the American Physiological Society*, 193, E1071-E1076.
25. Lewis, G.F., Carpentier, A., Adeli, K. & Giacca, A. (2002). Disordered fat storage and mobilization in the

pathogenesis of insulin resistance and type 2 diabetes.

Endocrine Reviews, 23, 201-229.

26. McGarry, J.D. (2001). Banting Lecture 2001:

Dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes*, 51, 7-18.

27. National Institute of Diabetes and Digestive and Kidney Diseases. (1996). Statistics related to overweight and obesity. *NIH Publications*, 96-4158.

28. Nisoli, E., Carruba, M.O., Tonello, C., Macor, C., Federspil, G. & Vettor, R. (2000). Induction of fatty acid translocase/CD36, peroxisome-proliferator-activated receptor- γ 2, leptin, uncoupling proteins 2 and 3, and tumor necrosis factor- α gene expression in human subcutaneous fat by lipid infusion. *Diabetes*, 49, 319-324.

29. Peterson, K.F. & Shulman, G.I. (2002). Pathogenesis of skeletal muscle insulin resistance in Type 2 diabetes mellitus. *The American Journal of Cardiology*, 90 (suppl.), 11G-18G.

30. Pi-Sunyer, F.X. (2002). The obesity epidemic: Pathophysiology and consequences of obesity. *Obesity Research*, 10, 97S-104S.

31. Ribiero, M.O., Carvalho, S.D., Schultz, J.J., Chiellini, G., Scanlan, T.S., Bianco, A.C. & Brent, G.A. (2001). Thyroid hormone-sympathetic interaction and adaptive thermogenesis are thyroid hormone receptor isoform-specific. *The Journal of Clinical Investigation*, 108, 97-105.
32. Rippe, J.M., McInnis, K.J. and Melanson, K.J. (2001). Physician involvement in the management of obesity as a primary medical condition. *Obesity Research*, 9 (suppl. 4).
33. Rothman, D.L. (2001). Studies of metabolic compartmentation and glucose transport using *in vivo* MRS. *NMR in Biomedicine*, 14, 149-160.
34. Ryder, J.W. et al. (1999). *In vitro* analysis of the glucose-transport system in GLUT4-null skeletal muscle. *Biochemistry Journal*, 342, 321-328.
35. Standaert, M.L., Ortmeyer, H.K., Sajan, M.P., Kanoh, Y., Bandyopadhyay, G., Hansen, B.C. & Farese, R.V. (2002). Skeletal muscle insulin resistance in obesity-associated type 2 diabetes in monkeys is linked to a defect in insulin activation of protein kinase C-(ζ/λ). *Diabetes*, 51, 2936-2943.

36. Silva, J.E., Leonard, J.L., Crantz, F.B. & Larsen, P.R. (1982). Evidence for two tissue-specific pathways for *in vivo* thyroxine 5'-deiodination in the rat. *Journal of Clinical Investigation*, 69, 1176-1184.
37. Torrance, C.J., Devente, J.E., Jones, J.P. and Dohm, G.L. (1997). Effects of thyroid hormone on GLUT4 glucose transporter gene expression and NIDDM in rats. *Endocrinology*, 138, 1204-1214.
38. Tremblay, F., Lavigne, C., Jacques, H. and Marette, A. (2001). Defective insulin-induced GLUT4 translocation in skeletal muscle in high fat-fed rats is associated with alterations in both Akt/Protein Kinase B and atypical protein kinase C (ζ/λ) activities. *Diabetes*, 50, 1901-1910.
39. Troiano, R.P., Flegal, K.M., Kuczmarski, R.J., Campbell, S.M. and Johnson, C.L. (1995). Overweight prevalence and trends for children and adolescents: The National Health and Nutrition Examination Surveys. *Archives of Pediatric Adolescent Medicine*, 140, 1085-1091.
40. Watson, R.T. and Pessin, J.E. (2001). Intracellular organizations of insulin signaling and GLUT4

translocation. *Recent Progress in Hormone Research*, 175-194.

41. Wilcox, H.G., Frank, R.A., and Heimberg, M. (1991). Effects of thyroid status and fasting on hepatic metabolism of apolipoprotein A-I. *Journal of Lipid Research*, 32, 395-405.

42. Zhou, H., Tran, P.O., Yang, S., Zhang, T., LeRoy, E., Oseid, E. and Robertson, R.P. (2004). Regulation of α -cell function by the β -cell during hypoglycemia in Wistar rats: the switch-off hypothesis. *Diabetes*, 53, 1482-1487.